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INFLUENCE OF CULTIVAR, EXPLANT SOURCE AND PLANT GROWTH REGULATOR ON CALLUS INDUCTION AND PLANT REGENERATION OF *CANNABIS SATIVA* L.

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The effects of different combinations of plant growth regulators (PGRs) on callus induction and plant regeneration were investigated in five cultivars of *Cannabis sativa* L. Callus was induced from different explant sources (young leaves, petioles, internodes, axillary buds) on MS basal medium with various concentrations of PGRs (2,4-D, DICAMBA, KIN, NAA). The highest frequency of callus induction (avg. 82.7% of eight medium combinations) was exhibited by petiole explants of cv. Fibrimon-24. Plant regeneration was obtained from all studied cultivars. The highest number of plants was regenerated from callus tissue of petiole explants on MS medium containing DICAMBA. A total of 46 plants (1.35% of callus) were regenerated: 16 (0.47%) from cv. Silesia petioles, 7 (0.20%) from cv. Novosadska petioles, 6 (0.18%) from cv. Fedrina-74 petioles, 12 (0.35%) from cv. Fibrimon-24 axillary buds, and 5 (0.15%) from cv. Juso 15 internodes. Significant improvement of hemp plant regeneration in vitro was achieved.

Key words: *Cannabis sativa*, axillary buds, callus induction, plant growth regulators, internode, leaf, petiole, plant regeneration.

INTRODUCTION

Hemp (*Cannabis sativa* L., Cannabaceae) is an important plant in medicine and pharmacy (Walsh et al., 2003). Fibers are used as raw material for paper and textile production (Alden et al., 1998). Recently there has been increased interest in transformation of *Cannabis sativa* to produce plants with enhanced fiber elasticity. Transgenic hemp can be used for production of biodegradable plastics (polyhydroxybutyrate) and other biopolymers as an alternative to plastics and glass fiber. Flax fibers can also be used to produce fully biodegradable composites (Tserki et al., 2005). A plant regeneration system is required to develop transgenic plants.

There are not many studies on tissue culture of hemp. Hemphil et al. (1978), Fisse et al. (1981), MacKinnon et al. (2000) and Feeney and Punja (2003) reported that callus readily produced roots but was unreceptive to shoot formation. Mandolino and Ranalli (1999) reported occasional shoot regeneration from callus. Successful regeneration and propagation has been achieved in other fibrous plants such as sisal (Das, 1992; Hazra et al., 2002) and flax (Rakouský et al., 1999; Yildiz and Ozgen, 2004). Conventional breeding and biotechnological approaches, including tissue culture and transformation procedures, could be extended to hemp breeding.

This study was intended to determine the optimal combinations of plant growth regulators for callus induction and plant regeneration of five hemp cultivars, using different types of explants.

MATERIALS AND METHODS

Seeds of five cultivars of *Cannabis sativa* L. were obtained from the Institute of Natural Fibres (Poznań, Poland): Silesia, Fibrimon-24, Novosadska, Juso-15 and Fedrina-74. Seedlings measuring ~10 cm, grown in a growth chamber at 22˚C under a 12 h photoperiod were sterilized in 5% calcium hypochlorite for 6, 8 and 15 min and thoroughly rinsed in sterile water. Explants

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Abbreviations: 2,4-D – dichlorophenoxyacetic acid; KIN – 6-furfurylaminopurine; NAA – 1-naphthaleneacetic acid; DICAMBA – 3,6-dichloro-o-anisic acid.

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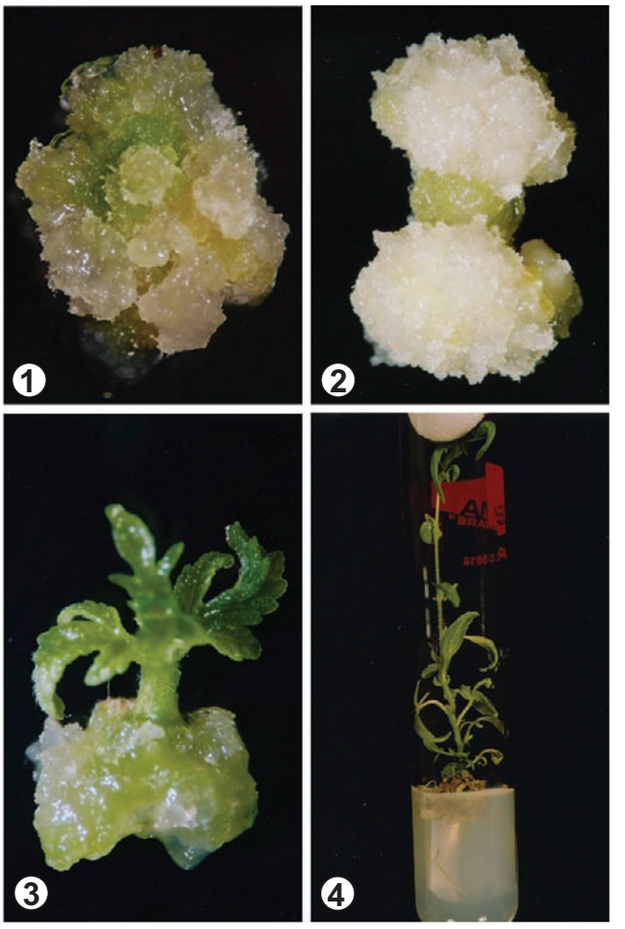
of young leaves, petioles, internodes and axillary buds were implanted in Petri dishes (90×15 mm) containing MS basal medium (Murashige and Skoog, 1962) supplemented with various concentrations of plant growth substances: 2,4-D, DIC, KIN and NAA (Tab. 1). Growth regulators were added after the media were autoclaved. Cultures were kept in darkness at 24˚C for 2–3 weeks. Callus was excised from the original explants, cut into ~0.5 cm^3 pieces and transferred every 3 weeks on the same fresh medium and incubated in a growth chamber at 22˚C under a 16 h photoperiod (~2000 lx). For root formation, regenerated plantlets ~2 cm high were excised from callus and cultured on MS basal medium supplemented with 1.0 mg l^{-1} IAA and 1.0 mg l^{-1} NAA. Rooted plants were transferred to soil and grown in a greenhouse.

TABLE 1. Eight combinations of KIN, NAA, 2,4-D, DICAMBA in MS medium used for callus formation and plant regeneration of *Cannabis sativa* L.

Ordinal number of plant growth regulator combination	KIN (mg l^{-1})	NAA (mg l^{-1})	2,4-D (mg l^{-1})	DICAMBA (mg l^{-1})
1	1.0	0.5	-	-
2	2.0	0.5	-	-
3	4.0	2.0	-	-
4	-	1.0	2.0	-
5	-	2.0	4.0	-
6	-	-	2.0	-
7	-	-	-	2.0
8	-	-	-	3.0



Figs. 1–4. Induction of callus, shoot and plant regeneration in *Cannabis sativa* cv. Silesia. **Fig. 1.** Induction of callus from leaf explants on MS medium containing 1.0 mg l^{-1} KIN + 0.5 mg l^{-1} NAA, after 3 weeks of culture. × 6. **Fig. 2.** Induction of callus from petiole explants on MS medium containing 3.0 mg l^{-1} DICAMBA, after 3 weeks of culture. x 6. **Fig. 3.** Shoot formation from callus of petiole after 6 weeks of culture. × 6. **Fig 4.** Rooting of shoot regenerated in vitro on MS medium with 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} NAA.

TABLE 2. Analysis of variance for effect of growth regulators on callus induction from *Cannabis sativa* L. explant sources

Source of variation	Degrees of freedom	Mean square for percentage of explants forming callus			
		Young leaf	Petiole	Internode	Axillary bud
Cultivar (C)	4	9321.41*	8301.23*	3532.52*	8256.92*
Medium combination (M)	7	1521.80*	2473.25*	771.14*	906.59*
C × M interaction	28	429.41*	444.24*	740.34*	248.36*
Error	80	107.85	131.82	127.06	57.80

* – significant at p = 0.01

STATISTICS

Experiments involving four explants, five cultivars and eight medium combinations were carried out in a randomized complete block (RCB) design in three replicates. The effect of plant growth regulators on callus induction for the different explants (young leaves, petioles, internodes, axillary buds) was expressed as percentages. Two-way ANOVA was used to test the significance of effects of plant growth regulator, medium combination, and cultivar × medium interaction on callus induction for each of the explants. Before ANOVA the percentage data were arcsine transformation to normalize the distribution. The means for cultivars, media with all cultivars, and media with particular cultivars were compared by the least significant difference (LSD) test. The LSD values were calculated on the transformed scale and were applied to the transformed means.

RESULTS

Sterilization of explants was best achieved by stirring in 5% calcium hypochlorite solution for 15 min. Briefer sterilization was ineffective.

Callus was obtained from all types of explant of the five cultivars (Silesia, Fibrimon-24, Novosadska, Fedrina-74, Juso-15). Callus varied in character: friable, compact or watery, with color ranging from pale yellow to green and white. Leaf explants of the five cultivars produced light green, watery callus, or else yellowish compact and nodular callus (Fig. 1). The cultured internode and petiole explants showed growth of callus from the cut ends, which was yellowish, nodular and compact (Fig. 2). White, watery, friable callus on the complete surface of the internode was observed very frequently. The axillary bud explants formed nodular and yellowish callus with green centers.

Table 2 gives the results of two-way ANOVA for the differences between medium combinations and between cultivars with regard to frequency of callus induction from particular explants. All null hypotheses on the effect of medium combination, cultivar and explant were rejected at p = 0.01. The medium combination × cultivar interactions were significant for all explant sources, indicating that the cultivars differed in the various medium combinations.

Averaging the results from all medium combinations and all cultivars together indicates highly efficient callus induction from petioles (avg. 52.3%) and young leaves (avg. 51.1%). The response of internode and axillary bud explants was lower, 37.0% and 17.2%, respectively. The highest efficiency of callus induction was achieved with petiole explants of cv. Fibrimon-24 (avg. 82.7%, 60.5–100% depending on the medium combination), cv. Novosadska (avg. 75.1%, 37.5–100%), young leaf explants of cv. Silesia, (avg. 75.1%, 38.1–99.5%) and cv. Fibrimon-24, (avg. 71.9%, 36.8–100%) (Tabs. 3, 4). For all medium combinations and cultivars averaged together, the frequency of callus induction from internode explants was lower (37.0%), and lowest from axillary buds (17.2%) (Tabs. 5, 6).

For all explant sources taken together, the highest efficiency of callus production was noted in cv. Fibrimon-24 on three medium combinations: 2.0 mg l^{-1} DICAMBA; 3.0 mg l^{-1} DICAMBA; and 2.0 mg l^{-1} KIN + 0.5 mg l^{-1} NAA (Tab. 7). Generally the best medium combinations were 2.0 mg l^{-1} or 3.0 mg l^{-1} DICAMBA (Tabs. 3–6). The efficiency of callus production obtained on MS medium supplemented with 1.0 mg l^{-1} KIN + 0.5 mg 1^{-1} NAA, 2.0 mg 1^{-1} KIN + 0.5 mg 1^{-1} NAA, or 2.0 mg l^{-1} 2.4-D was high only for some explant sources and cultivars.

Medium combination and explant origin had important effects not only on callus initiation but also on plant regeneration (Fig. 5). In the five cultivars, plantlets were formed on the same medium as the callus tissue after 6 weeks of culture. Plantlets were regenerated from nodular callus with meristematic centers of petioles, internodes and axillary buds (Fig. 3).

Figure 5 presents the efficiency of plant regeneration from callus, by explant source and by medium combination. The highest percentage of plantlet regeneration was for petiole explants of three cultivars on media with 3.0 mg l^{-1} and 2.0 mg l^{-1} DICAMBA (2.5% and 2.3% for Silesia, 2.0% and 1.4% for Novosadska, 1.5% and 1.4% for Fedrina-74). On medium with 2.0 mg l^{-1} 2 ,4-D, only cv. Silesia produced plantlets from petiole explants. Cv. Juso-15 on media with 2.0 mg l^{-1} and 3.0 mg l^{-1} DICAMBA regenerated plantlets only

TABLE 3. Mean percentage of callus induction from young leaf explants, by cultivar and growth regulator combination

Growth regulator combination mg l^{-1}	Cultivar					Mean
	Silesia	Fibrimon-24	Novosadska	Fedrina-74	Juso-15	
1.0 KIN + 0.5 NAA	99.5*a*	90.0b	39.9*de*	13.9a	26.7ab	54.0bc
2.0 KIN + 0.5 NAA	67.3*c*	83.2*b*	87.8*ab*	7.2*a*	20.9*ab*	53.2*c*
4.0 KIN + 2.0 NAA	38.1*d*	50.4*c*	53.2*cde*	10.7*a*	17.2*b*	33.9*d*
1.0 NAA + 2.0 2,4-D	66.4*cd*	41.3*c*	27.9*e*	11.5*a*	25.7*ab*	34.6*d*
2.0 NAA + 4.0 2,4-D	62.7*cd*	36.8*c*	63.8*cd*	7.8*a*	33.9*ab*	40.9*d*
2.0 2,4-D	79.9*bc*	81.7*b*	100.0*a*	14.3*a*	39.9*ab*	63.2*ab*
2.0 DICAMBA	92.5*ab*	100.0*a*	76.1*bc*	17.5*a*	41.5*ab*	65.5*a*
2.0 DICAMBA	94.7*ab*	91.9*ab*	64.2*cd*	20.20*a*	47.2*a*	63.6*ab*
Mean	75.1*a*	71.9*ab*	64.1b	12.9*d*	31.5c	51.1

Means within a column followed by the same letter did not differ significantly at p = 0.05

TABLE 4. Mean percentage of callus induction from petiole explants, by cultivar and growth regulator combination

Growth regulator combination mg l^{-1}	Cultivar					Mean
	Silesia	Fibrimon-24	Novosadska	Fedrina-74	Juso-15	
1.0 KIN + 0.5 NAA	36.7*c*	100.0a	76.3bc	33.7bc	29.4ab	55.2bc
2.0 KIN + 0.5 NAA	22.7*c*	89.8*ab*	70.9*c*	29.8*c*	27.7*ab*	48.2*cd*
4.0 KIN + 2.0 NAA	17.8*c*	77.0*bc*	67.8*cd*	31.1*bc*	15.9*b*	41.9*de*
1.0 NAA + 2.0 2,4-D	0.0*d*	77.1*bc*	37.5*d*	37.8*bc*	10.3*b*	32.5*e*
2.0 NAA + 4.0 2,4-D	0.0*d*	60.5*c*	55.1*cd*	46.0*bc*	21.1*ab*	36.5*e*
2.0 2,4-D	33.3*c*	89.1*ab*	97.9*ab*	22.9*c*	30.5*ab*	54.7*bc*
2.0 DICAMBA	96.0*a*	91.5*ab*	100.0*a*	80.3*a*	50.4*a*	83.6*a*
2.0 DICAMBA	67.3*b*	76.3*bc*	95.7*ab*	61.3*ab*	30.1*ab*	66.1*b*
Mean	34.2*c*	82.7a	75.1a	42.9*b*	26.9*c*	52.3

Means within a column followed by the same letter did not differ significantly at p = 0.05

TABLE 5. Mean percentage of callus induction from internode explants, by cultivar and growth regulator combination

Growth regulator combination mg l^{-1}	Cultivar					Mean
	Silesia	Fibrimon-24	Novosadska	Fedrina-74	Juso-15	
1.0 KIN + 0.5 NAA	98.1*a*	51.6a	62.5*bc*	22.7*abc*	19.0	50.8*a*
2.0 KIN + 0.5 NAA	71.1*b*	49.9*a*	56.3*c*	18.7*abc*	17.8	42.8*abc*
4.0 KIN + 2.0 NAA	66.0*bc*	11.1*b*	32.1*c*	11.4*c*	4.7	25.1*e*
1.0 NAA + 2.0 2,4-D	37.8*cd*	28.9*ab*	41.3*c*	14.8*bc*	3.3	25.2*e*
2.0 NAA + 4.0 2,4-D	30.7*d*	31.4*ab*	39.0*c*	25.7*abc*	12.7	27.9*de*
2.0 2,4-D	0.0*e*	50.0*a*	87.2*a*	39.9*ab*	23.4	40.1*bcd*
2.0 DICAMBA	29.9*d*	54.1*a*	90.2*a*	44.2*a*	21.5	48.0*ab*
2.0 DICAMBA	41.7*bcd*	0.0*c*	77.3*ab*	45.5*a*	16.7	36.2*cde*
Mean	46.9*b*	34.6e	60.7a	27.9*c*	14.9d	37.0

Means within a column followed by the same letter did not differ significantly at p = 0.05

from internode callus. On three medium combinations (0.5 mg l^{-1} NAA + 1.0 mg l^{-1} KIN; 2.0 mg l^{-1} 2,4-D; 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} NAA) Fibrimon-24 plantlets were induced only from axillary bud callus; the percentage was highest (2.3%) on medium containing 0.5 mg l^{-1} NAA + 1.0 mg l^{-1} KIN. Leaf callus of the tested cultivars formed only roots, and no plantlets were obtained. A total of 46 plants (1.4% of calluses) were regenerated: 16 (0.5%) from cv. Silesia petioles, 7 (0.2%) from cv. Novosadska petioles, 6 (0.2%) from

TABLE 6. Mean percentage of callus induction from axillary bud explants, by cultivar and growth regulator combination

Growth regulator combination mg l^{-1}	Cultivar					Mean
	Silesia	Fibrimon-24	Novosadska	Fedrina-74	Juso-15	
1.0 KIN + 0.5 NAA	9.7*ab*	67.6bc	34.7*a*	3.8*bc*	11.2*a*	25.4*a*
2.0 KIN + 0.5 NAA	3.5*bc*	81.3*ab*	5.2*bc*	9.6*ab*	13.5*a*	22.6*a*
4.0 KIN + 2.0 NAA	0.0*c*	35.9*de*	3.9*bc*	0.0*c*	0.0*c*	8.0*b*
1.0 NAA + 2.0 2,4-D	11.2*ab*	27.7*e*	0.0*c*	4.2*bc*	0.0*c*	8.6*b*
2.0 NAA + 4.0 2,4-D	0.0*c*	47.0*de*	0.0*c*	13.4*a*	0.0*c*	12.1*b*
2.0 2,4-D	15.3*a*	78.0*ab*	9.6*b*	15.2*a*	5.4*bc*	24.7*a*
2.0 DICAMBA	8.9*ab*	92.0*a*	11.1*b*	0.0*c*	9.8*a*	24.4*a*
2.0 DICAMBA	9.8*ab*	48.1*cd*	0.0*c*	0.0*c*	2.3*bc*	12.0*b*
Mean	7.3*b*	59.7*a*	8.1*b*	5.8*b*	5.3b	17.2

Means within a column followed by the same letter did not differ significantly at p = 0.05

TABLE 7. Mean percentage of callus production from all types of explants, by cultivar and growth regulator combination

Growth regulator combination mg l^{-1}	Cultivar					Mean
	Silesia	Fibrimon-24	Novosadska	Fedrina-74	Juso-15	
1.0 KIN + 0.5 NAA	43.95*ab*	58.70b	47.65b	32.48*a*	26.37a	41.83*b*
2.0 KIN + 0.5 NAA	46.30*ab*	75.22*a*	66.63*a*	13.56*b*	18.48*ab*	44.04*b*
4.0 KIN + 2.0 NAA	32.80*bc*	63.15*b*	39.80*b*	17.23*b*	15.43*c*	33.68*c*
1.0 NAA + 2.0 2,4-D	34.78*bc*	42.17*c*	25.15*c*	14.54*b*	11.53*c*	25.64*d*
2.0 NAA + 4.0 2,4-D	26.60*c*	39.59*c*	44.10*b*	18.68*b*	15.45*c*	28.89*d*
2.0 2,4-D	29.62*c*	63.34*b*	59.13*a*	21.60*b*	18.88*bc*	38.51*bc*
2.0 DICAMBA	49.67*a*	76.39*a*	67.35*a*	32.65*a*	31.65*a*	51.54*a*
2.0 DICAMBA	51.35*a*	76.81*a*	65.93*a*	35.02*a*	26.93*ab*	51.21*a*
Mean	39.38*c*	61.92*a*	51.97*b*	23.22*d*	20.59*d*	

Means within a column followed by the same letter did not differ significantly at p = 0.05

cv. Fedrina-74 petioles, twelve (0.4%) from cv. Fibrimon-24 axillary buds, and five (0.2%) from cv. Juso-15 internodes.

For rooting, plantlets were transferred to MS basal medium supplemented with 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} NAA, and root formation was noted 3 weeks later (Fig. 4). Of the 45 plantlets, 32 (69.9%) formed roots (13 Silesia, 8 Fibrimon-24, 5 Novosadska, 4 Fedrina-74, 2 Juso-15). The plantlets with roots were transferred to pots and grown in a growth chamber.

DISCUSSION

In this study, explants were derived from plants growing in pots, because preliminary experiments showed that hemp seeds are highly contaminated. Saeed et al. (1997) reported a similar problem with contamination of cotton seeds by large numbers of fungus spores and bacteria.

We studied the effects of various concentrations of plant growth substances on callus induction and plant regeneration from different explant sources (leaf, petiole, internode, axillary bud). Callus was obtained from all four types of explant of the five investigated cultivars, but the highest induction was from leaves and petioles. In experiments with hemp leaves, petioles, stems and cotyledons, Feeney and Punja (2003) obtained abundant callus from stem and leaf explants of three studied cultivars, and less callus formation from cotyledons. Fisse et al. (1981) and Mandolino and Ranalli (1999) reported that cotyledon and root explants did not produce callus well. We observed low frequency of callus from axillary buds and internodes. Similarly, Xie and Hong (2001) reported low efficiency of callus induction from internode explants of *Acacia mangium*.

In our experiments, hemp regenerants were obtained from internode-, petiole- and axillary bud-derived callus. Mandolino and Ranalli (1999) demonstrated occasional shoot regeneration of hemp from leaf callus. Hemphill et al. (1978), Fisse et al. (1981) and MacKinnon et al. (2000) described root development but no shoot formation from callus. Richez-Dumanois et al. (1986) induced direct multiplication of shoots from apical and axillary bud explants.

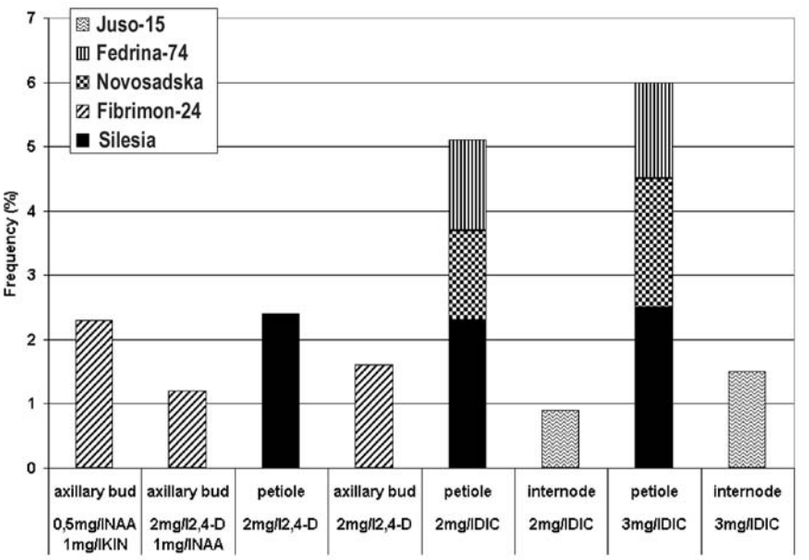


Fig. 5. Efficiency of plant regeneration from callus obtained from various explant sources of *Cannabis sativa* L. on MS medium supplemented with growth regulators.

In the tested cultivars of *Cannabis sativa*, high efficiency of callus induction and root formation from leaf explants was observed, but no plantlets were regenerated. Similarly, Feeney and Punja (2003) described only root production from leaves. In contrast, plant regeneration from leaf callus has been obtained in *Hypericum perforatum* (Pretto and Santarém, 2000), *Agave sisalana* (Hazra et al., 2002) and *Citrus grandis* (Tao et al., 2002). Direct multiplication of shoots from leaf without callus has been described in, for example, *Eucalyptus gunnii* (Hervé et al., 2001) and *Limonium altaica* (Jeong et al., 2001).

Medium combination and explant source had an important influence on callus initiation and plant regeneration. This study used supplemented MS basal medium for callus induction and plantlet regeneration in *Cannabis sativa*. The highest regeneration frequency was from petiole explants on medium supplemented with DICAMBA (2.0 mg l^{-1} or 3.0 mg l^{-1}). Medium with DICAMBA induced only callus from leaf explants, and medium containing 2,4-D and NAA initiated only roots from leaf callus. Tao et al. (2002) reported that only callus developed from leaf explants of *Citrus grandis* on medium with DICAMBA. Feeney and Punja (2003) obtained only roots from hemp leaf callus on MS medium with 2,4-D and NAA.

Among the combinations of auxin and cytokinin we tested, 0.5 mg l^{-1} IAA + 1.0 mg l^{-1} KIN yielded a superior response from axillary bud explants of cv. Fibrimon-24. KIN and NAA have been shown to be an efficient combination for induction of organogenesis of *Coleus forskohlii* from axillary buds and leaves (Reddy et al., 2001). In that study, 69.6% plant rooting was induced on MS basal medium containing 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} NAA. Various growth regulators have been used for root formation in several species: on MS medium with NAA + IBA, for example, 90.0% of *Citrus grandis* shoots (Tao et al., 2002) and 81.0% of *Iphigenia indica* shoots developed roots (Mukhopadhyay et al., 2002). In contrast, Sharma et al. (1991) and Reddy et al. (2001) found that auxin was not necessary for root induction in *Coleus forskohlii*. The maximum frequency of root formation (80.0%) of *Coleus forskohlii* were achieved on half-strength MS medium without growth regulators (Reddy et al., 2001). Minocha (1987) suggested that root formation on auxin-free medium may be due to the availability of higher-quantity endogenous auxin in shootlets raised in vitro.

In the present study, high efficiency of callus induction was achieved in four explant sources of five hemp cultivars by the application of plant growth regulator combinations. Feeney and Punja (2003) obtained high production of callus from stem and leaf explants of three hemp varieties. Up to now only Mandolino and Ranalli (1999) have described poor regeneration of plantlets from hemp. In our experiments there was some improvement of plantlet regeneration by organogenesis via callus in three explant sources from all five tested cultivars, but the efficiency of plantlet regeneration was low (1.4% of plated callus). Further experiments are needed. Successive modifications of media may lead to development of a more efficient plant regeneration system that can be used for production of transgenic hemp plants.

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